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Ramaria flavescentoides sp. nov. with clamped basidia from Pakistan

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Abstract—During an investigation of macrofungi from Pakistan, a new species with clamped basidia within *Ramaria* subg. *Laeticolora* and associated with *Abies pindrow* was found, which is proposed here as *Ramaria flavescentoides*. The new fungus morphologically resembles *R. flavescens* and clusters phylogenetically within the same clade. *Ramaria flavescentoides* is characterized by an obovate to obconic basidioma with pale orange to light orange branches and apices that are tinged light greenish grey, a monomitic hyphal system, ampulliform clamp connections, stellate crystals distributed within the upper branches, multiguttulate rhizomorphic strands, and oblong to ellipsoid verrucose basidiospores.

Key words—Himalayan moist temperate forest, ITS region, molecular identification, polychotomous branching

Introduction

Ramaria Fr. ex Bonord. (*Ramariaceae*) is a widespread non-gilled basidiomycete genus comprising nearly 220 species worldwide (Kirk & al. 2008). The basidiomata, which generally repeatedly branch dichotomously, are usually divided into stipe, branches, and apices. Important diagnostic characters include color, stipe consistency (fibrous vs gelatinous), odor, staining reactions to reagents, presence or absence of clamp connections, and spore size and ornamentation (Exeter & al. 2006). Villegas & al. (1999)

placed *Ramariaceae* in *Gomphales*, a placement also supported by molecular phylogenetic studies (Hibbett & al. 1997, Bruns & al. 1998, Humpert & al. 2001, Giachini 2004). *Ramaria,* which includes both saprobic and ectomycorrhizal species, is currently divided into four subgenera: *R.* subg. *Echinoramaria* Corner, *R.* subg. *Lentoramaria* Corner, *R.* subg. *Ramaria,* and *R.* subg. *Laeticolora* Marr & D.E. Stuntz (Marr & Stuntz 1973, Exeter & al. 2006). Previously these divisions were based on spore ornamentation, monomitic vs. dimitic rhizomorphic strands, humicolous vs. lignicolous substrate, and presence or absence of clamp connections.

Ramaria flavescentoides is described here based on morphological and molecular data as a new species in *R.* subg. *Laeticolora*. Both *R.* subg. *Laeticolora* and *R.* subg. *Ramaria* tend to develop larger fruiting bodies than *R.* subg. *Echinoramaria* and *R.* subg. *Lentoramaria*. *Ramaria* subg. *Laeticolora* is further characterized by non-striate basidiospores that are almost smooth to distinctly ornamented at 1000× (vs. striate in *R.* subg. *Ramaria*), tissues that are primarily non-amyloid (vs. amyloid in *R.* subg. *Ramaria*), single to fasciculate stipe bases, usually brightly colored branches and apices, and a terrestrial habit (Exeter & al. 2006).

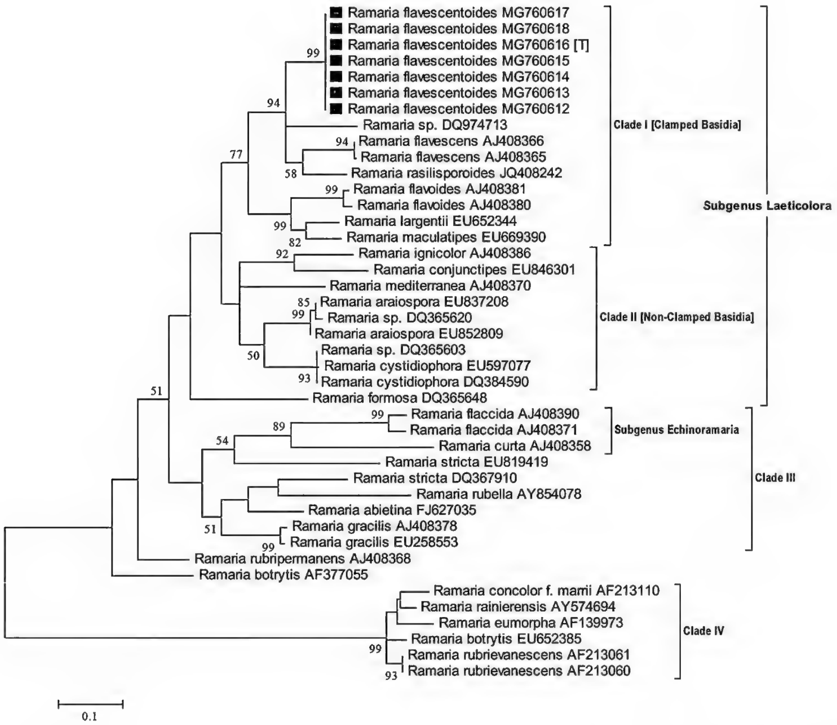
Materials and methods

Collections

The sampling site was selected based on the richness and abundance of aphyllophoralean fungi in an area of probable high basidiomycete biodiversity in a moist temperate coniferous forest in Khyber Pakhtunkhwa, Pakistan where air temperatures range from –4 to 25°C, and the soil is loamy with gravel and variably sized stones. Field notes and photographs were made of all collections, and the specimen was morphologically characterized following Corner (1950). Features (stipe, branches, rhizomorphic hyphae) were measured microscopically at 1000× using a compound microscope equipped with an ocular micrometer. Measurements of basidiospores, basidia, cystidia, and rhizomorphic hyphae were determined from at least 20 measurements per character. Small (~1 cm) portions of the hymenium were stored in 2% CTAB buffer in 1.5 ml Eppendorf vials and stored at –20°C for molecular analysis; the remaining material was dried using a fan heater and stored in sealed plastic bags. The collections are curated at the mycological herbarium at the University of the Punjab, Lahore, Pakistan (LAHMH).

DNA extraction, PCR amplification & sequencing

Hymenial tissue was removed with sterile forceps for DNA extraction by a CTAB method following Gardes & Bruns (1993), modified for Gene-Clean silica emulsion binding and purification. The polymerase chain reaction (PCR) followed Gardes &


■ Ramaria flavescentoides MG760617
■ Ramaria flavescentoides MG760618
■ Ramaria flavescentoides MG760616 [T]
■ Ramaria flavescentoides MG760615
■ Ramaria flavescentoides MG760614
■ Ramaria flavescentoides MG760613
■ Ramaria flavescentoides MG760612
Ramaria sp. DQ974713
Ramaria flavescens AJ408366
Ramaria flavescens AJ408365
Ramaria rasilisporoides JQ408242
Ramaria flavoides AJ408381
Ramaria flavoides AJ408380
Ramaria largentii EU652344
Ramaria maculatipes EU669390
Ramaria ignicolor AJ408386
Ramaria conjunctipes EU846301
Ramaria mediterranea AJ408370
Ramaria araiospora EU837208
Ramaria sp. DQ365620
Ramaria araiospora EU852809
Ramaria sp. DQ365603
Ramaria cystidiophora EU597077
Ramaria cystidiophora DQ384590
Ramaria formosa DQ365648
Ramaria flaccida AJ408390
Ramaria flaccida AJ408371
Ramaria curta AJ408358
Ramaria stricta EU819419
Ramaria stricta DQ367910
Ramaria rubella AY854078
Ramaria abietina FJ627035
Ramaria gracilis AJ408378
Ramaria gracilis EU258553
Ramaria rubripermanens AJ408368
Ramaria botrytis AF377055
Ramaria concolor f. marrii AF213110
Ramaria rainierensis AY574694
Ramaria eumorpha AF139973
Ramaria botrytis EU652385
Ramaria rubrievanescens AF213061
Ramaria rubrievanescens AF213060
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Clade I [Clamped Basidia]
Subgenus Laeticolora
Clade II [Non-Clamped Basidia]
Subgenus Echinoramaria
Clade III
Clade IV
0.1

Fig. 1. Maximum Likelihood phylotree of the evolutionary history of ITS sequences of *Ramaria* species, including *R. flavescentoides*. The tree with the highest log likelihood (–10063.0715) is shown, drawn to scale with branch lengths indicating the number of substitutions per site. Bootstrap support is shown next to the branches.

Bruns (1993), using the fungus-specific ITS1F primer (CTTGGTCATTTAGAGGAAGT) and the eukaryotic ITS4 primer (TCCTCCGCTTATTGATATGC) to amplify the nuclear rDNA-ITS region. The hot-start enzyme JumpStart catalyzed the PCR with 2 min at 94°C; followed by 30 cycles of 30 s at 94°C, 30 s at 53°C, and 40 s + 5 s per cycle at 72°C; and finishing with 5 min at 72°C. The PCR products were purified with QIAquick, sequenced bi-directionally using the reverse and forward primers and BigDye 3.1 on an ABI 3730 DNA sequencer, and edited in Sequencher 4.5 by Jodrell Laboratory, Royal Botanical Gardens, Kew, UK. The DNA sequences were BLAST searched at NCBI (https://www.ncbi.nlm.nih.gov/) and submitted to GenBank. Sequences were aligned and corrected manually for phylogenetic analysis with MacClade 4.08. Percentage identities were calculated using DNAStar software.

Phylogenetic analysis

The rDNA ITS sequences were aligned with sequences of other *Ramaria* species selected from GenBank. The original dataset comprising 1108 characters including gaps was trimmed by 252 characters excluded from 3′ and 5′ ends of the sequences. All gaps were treated as missing data. Of the 856 characters (of 'unord' type and equally weighted) analysed, 202 characters were constant, 616 were parsimony-uninformative, and 507 were parsimony-informative. The Branch-and-Bound search method was used for bootstrapping with 1000 bootstrap replicates in MEGA5 (Tamura & al. 2011).

Results

ITS rDNA-based identification and phylogenetic analysis

The amplification of the rDNA ITS from *Ramaria flavescentoides* produced 630–665 bp fragments. The consensus sequence of *R. flavescentoides* showed 91% identity and 65% query coverage with an undescribed *Ramaria* sp. (DQ974713.1) and 88% identity and 75% query coverage with *R. flavescens* (AJ408365).

Species included in this study were selected from all four *Ramaria* subgenera based on the diagnostic characters of each subgenus. The maximum likelihood phylogram represents four clades (Fig. 1). Clade I represents species belonging to *R.* subg. *Laeticolora* with clamped basidia, Clade II represents species belonging to *R.* subg. *Laeticolora* with non-clamped basidia, and Clades III and IV represent species belonging to *R.* subg. *Echinoramaria*, *R.* subg. *Lentoramaria,* and *R.* subg. *Ramaria.* The Clade I sequences from *R. flavescentoides* clustered with those from *R. flavescens*, *R. flavoides*, *R. largentii*, *R. maculatipes*, *R. rasilisporoides*, and *Ramaria* sp. (bootstrap = 77%). Within the clade, *R. flavescentoides* was sister to *R. flavescens*, *R. rasilisporoides*, and *Ramaria* sp. (bootstrap = 94%). Percentages of genetic characters shared by *R. flavescentoides* with other species were: *Ramaria* sp.—83%, *R. flavescens*—82%, *R. largentii*—81%, *R. rasilisporoides*—79%, *R. maculatipes*—78%, and *R. flavoides*—77%.

Taxonomy

Ramaria flavescentoides Hanif & Khalid, **sp. nov.** Fig. 2

MB 823965

Differs from *Ramaria flavescens* by its obconic to obovate basidioma, its non-bulbous fasciculate stipe, and the presence of stellate crystals in the upper branches.

Type: Pakistan. Khyber Pakhtunkhwa province, Hazara Division, Ayubia–Khanspur, 34°01′24″N 73°24′14″E, 2159 m asl, under *Abies pindrow*, 8 August 2009, M. Hanif MH88 (**Holotype**, LAHMH0889; GenBank MG760616).

Etymology—Named for its macroscopic resemblance with *R. flavescens.*

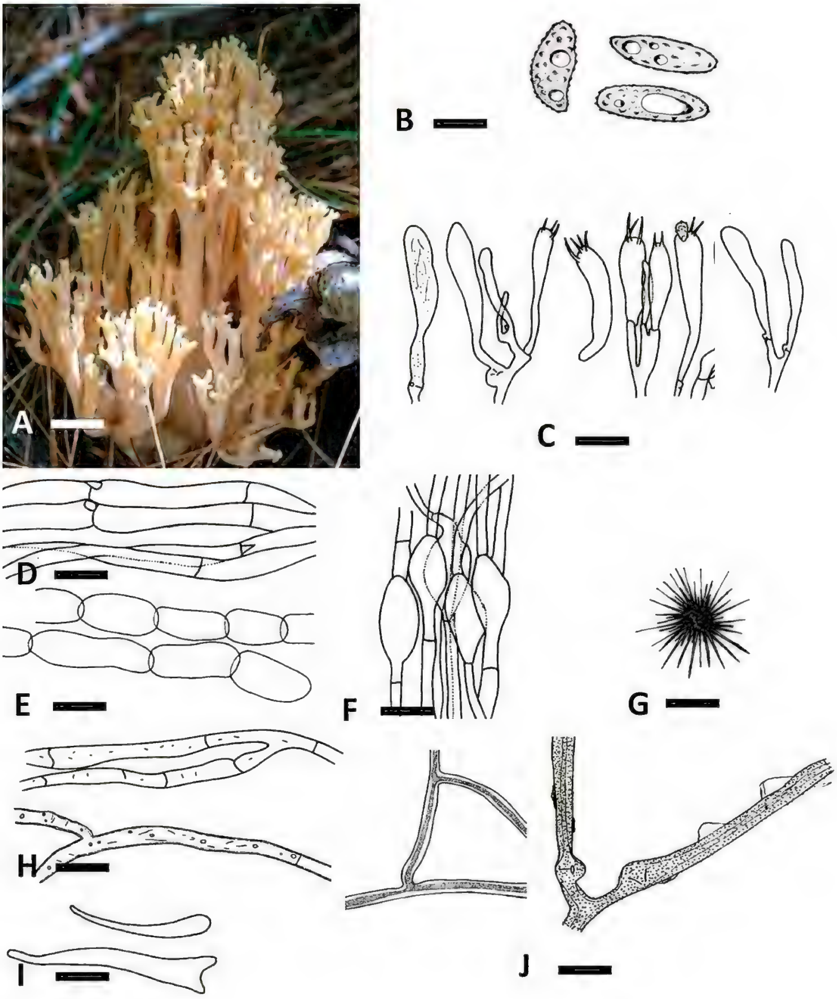

Fig. 2. *Ramaria flavescentoides* (holotype, LAHMH0889). A. Basidiocarps; B. Basidiospores; C. Basidia and cystidia; D. Stipe tramal hyphae; E. Overlapped cellular hyphae of upper branches; F. Ampulliform tramal hyphae of upper branches; G. Stellate crystals among stipe hyphal mass; H. Cellular hyphae of upper branches; I. Tramal elements of upper branches; J. Dimitic hyphae of rhizomorph. Scale bars: A = 2 cm; B, C = 3 µm; D, G = 15 µm; E, H = 17 µm; F = 13.5 µm; I = 6 µm; J = 37.5 µm.

Basidioma 13–15 cm high, ≤10 cm broad, repeatedly branched, usually obovate to obconic in outline; orange to light orange on drying. Stipe ≤3(–

4) cm high, compound to fasciculate in groups of 2–5, orange white at the base, upward pale orange, tapering gradually downward into a basal mycelial mat or white tomentum covering the stipe base. RHIZOMORPHS white, involving significant amounts of substrata, drying light orange. FLESH white, solid, gelatinous or slippery, dried flesh firm but easily penetrated. MAJOR BRANCHES 4–6, 1.5–2 cm thick, stout, more or less terete, ascending, orange white below, upward pale. BRANCHES 4–7 ranks, ascending, polychotomous, smooth and gelatinous from exterior, pale orange; axils more or less rounded. APICES 2–4 mm long, acute to blunt, light orange up to the tips with light greenish grey tint. TASTE, ODOR, and MACROCHEMICAL REACTIONS not recorded.

STIPE TRAMAL HYPHAE 6–11 µm wide, hyaline, thin-walled, parallel, slightly ampulliform, occasionally clamped, grainy encrusted with crystalline material of various shapes; gloeoplerous hyphae not observed. TRAMAL HYPHAE OF UPPER BRANCHES 6–8 µm diam., hyaline, thin-walled, parallel, ampulliform; skeletal generative hyphae present, clamps occasional, stellate crystalline material 32 µm diam. HYPHAE OF BASAL MAT monomitic, 3–4 µm diam., thin-walled, branched, clamped, 6 µm thick at clamps, grainy encrusted with crystalline material of various shapes; generative hyphae 7–10 µm diam. RHIZOMORPHIC HYPHAE smooth to encrusted, diameters vary. SUBHYMENIUM rudimentary, hyphal structure is monomitic with thin-walled and thick-walled hyphae of various diameters, overlapped, septate; frequent ampulliform hyphae (27 × 15 µm). HYMENIUM thick during maturation. BASIDIA 32–50(–70) × 9–12 µm, clavate, clamped; contents homogeneous when young, slightly grainy, hyaline or slightly yellowish, slightly cyanophilous, 4-spored; sterigmata 6–8 µm high. BASIDIOSPORES (10.7–)11–13(–15) × (4.8–)5–6(–7) µm, verrucose, multiguttulate when mature, oblong to ellipsoid, slightly curved at one end, contents weakly cyanophilous; wall moderately thick; ornamentation strongly cyanophilous, roughened outline of low coarse warts and delicate meandering ridges covering significant areas of wall.

HABITAT & DISTRIBUTION—Terrestrial, solitary, under *Abies pindrow*. Known only from the type area, Ayubia–Khanspur, fruiting in July–August at 1900–2200 m asl.

ADDITIONAL SPECIMENS EXAMINED—PAKISTAN. **KHYBER PAKHTUNKHWA PROVINCE, Hazara Division**, Ayubia–Khanspur, 34°01′24″N 73°24′14″E, 2159 m asl, 17 Aug 2009, M. Hanif MH136 (LAHMH1789.1; GenBank MG760618); Helipad, 34°01′31″N 73°25′19″E, 1974 m asl, 25 Jul 2008, M. Hanif MH253 (LAHMH2578.2; GenBank MG760612); M. Hanif MH254 (LAHMH2578.3; GenBank MG760617);

M. Hanif MH96 (LAHMH2578.5; GenBank MG760615); M. Hanif MH101 (LAHMH2578.1; GenBank MG760613); 16 Aug 2009, M. Hanif MH97 (LAHMH1689; GenBank MG760614).

Comments—*Ramaria flavescentoides* is characterized by medium sized obovate to obconic basidiomata with pale orange to light orange colored branches and lightly tinged greenish grey apices and a compound to fasciculate stipe that narrows gradually downward into a mat of white mycelia and rhizomorphic strands.

The new species resembles *R. flavescens* (Schaeff.) R.H. Petersen in shape, branching pattern, and basidioma color, also sharing a similar spore shape and ornamentation and the presence of basidial clamp connections. However, our specimens differ morphologically from *R. flavescens,* which produces smaller (10 × 7 cm) obpyriform profusely branched basidiomata with a rather abrupt ball surmounting the stipe and smaller basidiospores (11–12.5 × 4.8–5.6 µm; Petersen 1974). The presence of stellate crystalline material in the hyphae of upper branches is another important character of *R. flavescentoides.* Similar crystals have been reported by Zhang & al (2005) in the stipe and upper branches of *Ramaria luteoaeruginea* P. Zhang & Zhu L. Yang in *R.* subg. *Echinoramaria*. Phylogenetically, *R. flavescentoides* nests adjacent to *R. flavescens*; and *R. flavescentoides* and *R. flavescens* cluster together in clade I with other species having clamped basidia (Fig. 1).

Ramaria flavescentoides shares similar branching pattern, clamped basidia, warted (verrucose) spores with morphological similarities with its phylogenetic sister species, *R. rasilisporoides* Exeter, which clusters in the same clade with strong (94%) bootstrap support (Fig. 1). *Ramaria rasilisporoides* differs from *R. flavescentoides* in its pale yellow basidiomata, amyloid stipe tissues, deeply rooting stipe with numerous abortive branchlets, and smaller basidiospores (Exeter & al. 2006).

Ramaria mairei Donk, the only species of subg. *Laeticolora* previously reported from Pakistan (Ahmad 1972; Ahmad & al. 1997), is distinguished by larger (18 cm) basidiomata, a 8 cm high stipe, numerous pale or deep lemon to pale ochraceous and rather lax or fairly crowded branches, and slightly longer (10–15-µm) oblong to ellipsoid roughened pale ochraceous basidiospores (Ahmad 1972).

Phylogenetic analysis also supports the morphological identification. *Ramaria flavescentoides* clusters with the clamped species of subg. *Laeticolora*. This clade seems to be paraphyletic and originated with non-clamped species of this subgenus with unsupported bootstrap frequency.

Humpert & al. (2001) reported the genus *Ramaria* as polyphyletic, which our analyses support.

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